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International Preliminary Examining Authority
European Patent Office
Erhardtstrasse 27
D-80469 Munich
GERMANY

By facsimile & mail

YOUR REF

OUR REF P036148WO: HRG/RMB

5th October 2005

Dear Sirs,

Re: International Application No. PCT/IB2004/004335
University of Groningen *et al.*

I refer to the Written Opinion, dated 2nd May 2005, issued by the EPO acting as International Searching Authority for this application.

In response to the Written Opinion for this application, I now enclose a Demand for International Preliminary Examination of this application.

International Preliminary Examination should be based on the amended claims filed in connection with this application on 1st July 2005 under Article 19 PCT. I enclose a copy of these amended claims and of the accompanying letter to the International Bureau of WIPO dated 1st July 2005.

Yours truly,

S. E. KIRSCH

pp GOODFELLOW, HUGH ROBIN KIRSCH, SUSAN EDITH

Enc: PCT Demand and fee calculation sheet
Letter dated 1st July 2005 to the International Bureau of WIPO
Amended claims filed with letter dated 1st July 2005

FACSIMILE MESSAGE

To: **EPO Munich**
Fax No.: **00 49 89 2399 4465**

This fax consists of 18 sheets. If a sheet is missing or has been imperfectly received, please contact us immediately (Tel: 020-7242 8692; Fax: 020-7405 4166). If you are not the addressee, please contact us immediately and then destroy this fax.

The demand must be filed directly with the competent International Preliminary Examining Authority or, if two or more Authorities are competent, with the one chosen by the applicant. The full name or two-letter code of that Authority may be indicated by the applicant on the line below:

IPEA/ EP

PCT

CHAPTER II

DEMAND

under Article 31 of the Patent Cooperation Treaty:
The undersigned requests that the international application specified below be the subject of international preliminary examination according to the Patent Cooperation Treaty.

For International Preliminary Examining Authority use only

Identification of IPEA	Date of receipt of DEMAND

Box No. I IDENTIFICATION OF THE INTERNATIONAL APPLICATION

Applicant's or agent's file reference: P036148WO: HRG

International application No.	International filing date *(day/month/year)*	(Earliest) Priority date *(day/month/year)*
PCT/IB2004/004335	6 December 2004 (06/12/2004)	5 December 2003 (05/12/2003)

Title of invention: IMPROVED CYTOKINE DESIGN

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[X] Further applicants are indicated on a continuation sheet.

International application No.
PCT/IB2004/004335

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☒ Further applicants are indicated on another continuation sheet.

International application No.
PCT/IB2004/004335

Continuation of Box No. II APPLICANT(S)

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☒ Further applicants are indicated on another continuation sheet.

International application No.
PCT/IB2004/004335

Continuation of Box No. II APPLICANT(S)

If none of the following sub-boxes is used, this sheet should not be included in the demand.

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☐ Further applicants are indicated on another continuation sheet.

International application No.
PCT/IB2004/004335

Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The following person is ☒ agent ☐ common representative

and ☒ has been appointed earlier and represents the applicant(s) also for international preliminary examination.

☐ is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.

☐ is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

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☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No. IV BASIS FOR INTERNATIONAL PRELIMINARY EXAMINATION

Statement concerning amendments:*

1. The applicant wishes the international preliminary examination **to start on the basis of:**

☐ the international application as originally filed

the description ☒ as originally filed
☐ as amended under Article 34

the claims ☐ as originally filed
☒ as amended under Article 19 (together with any accompanying statement)
☐ as amended under Article 34

the drawings ☒ as originally filed
☐ as amended under Article 34

2. ☐ The applicant wishes any amendment to the claims under Article 19 to be considered as reversed.

3. ☐ The applicant wishes the start of the international preliminary examination **to be postponed** until the expiration of the applicable time limit under Rule 69.1(d).

4. ☐ The applicant expressly wishes the international preliminary examination **to start earlier** than at the expiration of the applicable time limit under Rule 54*bis*.1(a).

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Language for the purposes of international preliminary examination: English

☒ which is the language in which the international application was filed.
☐ which is the language of a translation furnished for the purposes of international search.
☐ which is the language of publication of the international application.
☐ which is the language of the translation (to be) furnished for the purposes of international preliminary examination.

Box No. V ELECTION OF STATES

The filing of this demand constitutes the election of all Contracting States which are designated and are bound by Chapter II of the PCT.

See Notes to the demand form

Sheet No. . 6

International application No.
PCT/IB2004/004335

Box No. VI CHECK LIST

The demand is accompanied by the following elements, in the language referred to in Box No. IV, for the purposes of international preliminary examination:

				For International Preliminary Examining Authority use only: received	not received
1. translation of international application	:		sheets	☐	☐
2. amendments under Article 34	:		sheets	☐	☐
3. copy (or, where required, translation) of amendments under Article 19	:	9	sheets	☐	☐
4. copy (or, where required, translation) of statement under Article 19	:	1	sheets	☐	☐
5. letter	:	1	sheets	☐	☐
6. other *(specify)*	:		sheets	☐	☐

The demand is also accompanied by the item(s) marked below:

1. ☒ fee calculation sheet
2. ☐ original separate power of attorney
3. ☐ original general power of attorney
4. ☐ copy of general power of attorney; reference number, if any:
5. ☐ statement explaining lack of signature
6. ☐ sequence listing in computer readable form
7. ☐ tables in computer readable form related to a sequence listing
8. ☐ other *(specify)*:

Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).

S. E. Kirsch

KIRSCH, SUSAN EDITH

GOODFELLOW, HUGH ROBIN

Authorised Representative

For International Preliminary Examining Authority use only

1. Date of actual receipt of DEMAND:

2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):

3. ☐ The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply.
   ☐ The applicant has been informed accordingly.
4. ☐ The date of receipt of the demand is WITHIN the time limit of 19 months from the priority date as extended by virtue of Rule 80.5.
5. ☐ Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82.
6. ☐ The date of receipt of the demand is AFTER the expiration of the time limit under Rule 54*bis*.1(a) and item 7 or 8, below, does not apply.
7. ☐ The date of receipt of the demand is WITHIN the time limit under Rule 54*bis*.1(a) as extended by virtue of Rule 80.5.
8. ☐ Although the date of receipt of the demand is after the expiration of the time limit under Rule 54*bis*.1(a), the delay in arrival is EXCUSED pursuant to Rule 82.

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Demand received from IPEA on:

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SUISSE

YOUR REF

OUR REF P036148WO:HRG/PHA

1st July 2005

Dear Sirs,

Re: International Application No. PCT/IB2004/004335
University of Groningen et al.

I refer to the amended claim set that was submitted on 29th June 2005 under Article 19 PCT. Unfortunately, it has come to my attention that this claim set contains some minor errors in claim dependencies. As the 2-month period provided by Rule 46(1) PCT has not yet expired, I therefore enclose an amended claim set to replace the claim set submitted on 29th June 2005. Please ignore my letter of 29th June and its enclosures. I apologise for any inconvenience caused.

As before, claims 63-66 are new. Claim 60 is amended so as to be dependent upon claims 58 and 59. Claim 28 is amended so as to be dependent on claims 26 and 27. Claims 29-32 are amended so as to be dependent on claim 28.

I hereby request that the enclosed claims be published pursuant to Rule 48.2(h) of the Implementing Regulations of the PCT.

Yours truly,

GOODFELLOW, HUGH ROBIN

Enc.

CONFIDENTIAL FACSIMILE MESSAGE

To: **World Intellectual Property**
Fax No.: **41227401435**

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CLAIMS

1. A β sheet multimeric cytokine whose sequence has been altered by mutating a residue in a monomer component of the multimeric cytokine protein so as to improve the free energy of the monomer or of the multimeric complex relative to the wild-type unmutated monomer component so as to be more stable than the wild-type, unaltered cytokine protein, wherein said mutated residue is non-conserved between homologous members of the cytokine family.

2. A cytokine according to claim 1, which is a member of the TNF ligand family.

3. A cytokine according to claim 2, which is TRAIL.

4. A cytokine according to claim 3, which is mutated in the soluble C-terminal portion of the molecule.

5. A cytokine according to any preceding claim, which is mutated at one or more of the following positions:

a) a non-conserved residue at the surface of the monomer component of the multimeric cytokine;

b) a non-conserved residue close to the interface between two of the monomer components of the multimeric cytokine;

c) for trimeric cytokines, a non-conserved residue along the central trimeric axis;

d) a miscellaneous residue whose mutation is energetically favourable.

6. A cytokine according to claim 5, which is mutated in the external loop that connects that C and D anti-parallel beta strands (the CD loop), following the notation according to Eck (Eck et al., J. Biol. Chem. 267, 2119-2122 (1992).

7. A cytokine according to claim 5 part a), which is mutated at one or both positions 194 and 196.

8. A cytokine according to claim 7, which is a TRAIL mutant containing the mutations E194I and/or I196S.

9. A cytokine according to claim 5 part b), which is mutated at one or more of the positions 125, 163, 185, 187, 232, 234, 237, 203, 205, 239, 241, 271, 274.

10. A cytokine according to claim 9, which is a TRAIL mutant containing one or more of the mutations D203I, Q205M and Y237F.

11. A cytokine according to claim 5 part c), which is mutated at one or more of positions 227, 230 and 240.

12. A cytokine according to claim 11, which is a TRAIL mutant containing the mutation R227M.

13. A cytokine according to claim 11, which is a TRAIL mutant containing the mutation C230S and Y240F.

14. A cytokine according to claim 5 part d), which is mutated at one or more of the positions 123, 272, 225, 280, 163, 123 and 208.

15. A cytokine according to claim 14, which is a TRAIL mutant containing the mutation S225A.

16. A cytokine which is mutated at more than one position as listed in claim 5, parts a) to d).

17. A cytokine according to claim 16, which is a TRAIL mutant containing the mutations E194I, I196S and S225A.

18. A cytokine according to any one of claims 1-17, wherein the described mutations are introduced into a soluble form of the cytokine.

19. A cytokine according to claim 18, which is a TRAIL mutant comprising residues 114-281.

20. A β sheet multimeric cytokine with selectivity for a target receptor, in which one or more amino acids in the cytokine that are located in the receptor-binding interface are substituted for replacement residues that include amino acid side-chain conformations that are predicted to fit into the binding interface with the target receptor so as to provide an increase in binding affinity and selectivity/specificity of the cytokine protein for that target receptor, provided that these are not residues interacting with amino acids that are conserved among receptors bound by the cytokine protein.

21. A β sheet multimeric cytokine according to claim 20 which has altered affinity for a particular target receptor.

22. A β sheet multimeric cytokine with selectivity for two or more target receptors wherein selectivity for a first target receptor is achieved by substituting one or more amino acids in the cytokine for replacement residues so as to decrease affinity for one or more different target receptors, provided that these are not residues interacting with amino acids that are conserved among receptors bound by the cytokine protein.

23. A cytokine according to claim 21 or claim 22, which is mutated at one or more of the positions 131, 269, 130, 160, 218, 220, 149, 155, 214, 195, 191 and 267 in the cytokine.

24. A cytokine according to any one of claims 20 to 23, which is a member of the TNF ligand family.

25. A cytokine according to claim 24, which is TRAIL.

26. A cytokine according to claim 25, which has superior selectivity for the DR5 (TRAIL-R2) or DR4 (TRAIL-R1) over the decoy receptors DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4).

27. A cytokine according to claim 25, which has superior selectivity for the death receptor 5 (TRAIL-R2) over selectivity for the death receptor 4 (TRAIL-R1).

28. A cytokine according to claim 26 or 27, which contains one or more of the mutations G131R, D269H, D269K, D269R, R130E, G160K, D218R, G160M, I220M, I220H, R149D, R149H, E155M, T214R, E195R, R191E and D267R.

29. A cytokine according to claim 28, which contains the mutations G160M or D269H.

30. A cytokine according to claim 28, which contains the mutations D269H and T214R.

31. A cytokine according to claim 28, which contains the mutations D269H and E195R.

32. A cytokine according to claim 28, which contains the mutations R191E and D267R.

33. A cytokine according to claim 25, which has superior selectivity for the death receptor 4 (TRAIL-R1) over selectivity for the death receptor 5 (TRAIL-R2).

34. A cytokine according to claim 33, which contains one or more of the mutations D218Y, D218E, D218K, D218H and D218F.

35. A β sheet multimeric cytokine with selectivity for a target receptor whose sequence has been altered by;

a) mutating a residue in a monomer component of the multimeric cytokine protein so as to improve the free energy of the monomer or of the multimeric complex relative to the wild-type unmutated monomer component, wherein said mutated residue is non-conserved between homologous members of the cytokine family, so as to be more stable than the wild-type, unaltered cytokine protein, and

b) substituting one or more amino acids in the cytokine that are located in the receptor-binding interface for replacement residues that include amino acid side-chain conformations that are predicted to fit into the binding interface with the target receptor so as to provide an increase in binding affinity and selectivity/specificity of the cytokine protein for that target receptor, provided that these are not residues interacting with amino acids that are conserved among receptors bound by the cytokine protein,

so as to provide variants with enhanced stability and increased binding affinity and selectivity/specificity for the target receptor.

36. A β sheet multimeric cytokine with selectivity for a target receptor whose sequence has been altered by;

a) mutating a residue in a monomer component of the multimeric cytokine protein so as to improve the free energy of the monomer or of the multimeric complex relative to the wild-type unmutated monomer component, wherein said mutated residue is non-conserved between homologous members of the cytokine family, so as to be more stable than the wild-type, unaltered cytokine protein, and

b) substituting one or more amino acids in the cytokine for replacement residues so as to decrease affinity for one or more different target receptors, provided that these are not residues interacting with amino acids that are conserved among receptors bound by the cytokine protein

so as to provide variants with enhanced stability and selectivity/specificity for the target receptor.

37. A cytokine according to claim 35 or 36, which is a member of the TNF ligand family.

38. A cytokine according to claim 37, which is TRAIL.

39. A cytokine according to claim 38, which contains the mutations D269H and T214R.

40. A cytokine according to claim 38, which contains the mutations D269H, E194I and I196S.

41. A computer-implemented method for the stabilisation of a β sheet multimeric cytokine, comprising the step of:

mutating a residue in a monomer component of the multimeric cytokine protein so as to improve the free energy of the monomer or of the multimeric complex relative to the wild-type unmutated monomer component;

wherein said mutated residue is non-conserved between homologous members of the cytokine family.

42. A method according to claim 41, wherein the non-conserved residue that is mutated is at the surface of the monomer component of the multimeric cytokine protein.

43. A method according to claim 41, wherein the non-conserved residue that is mutated is near a position close to the interface between two monomer components of the cytokine protein.

44. A method according to claim 41, wherein in a trimeric cytokine protein, the non-conserved residue that is mutated is at a position along the central trimeric axis of the multimeric protein.

45. A method according to any one of claims 41-44, wherein more than one non-conserved residue is mutated.

46. A method according to any one of the preceding claims, wherein non-conserved residues are identified using a computer-implemented alignment algorithm.

47. A method according to claim 64, wherein in an alignment between the candidate for mutation and other members of the same protein family, a conserved residue is one that is shared between at least 50% of the family.

48. A method according to any one of the preceding claims, wherein a protein design algorithm is used to facilitate the identification of candidate residues for mutation.

49. A method according to claim 48, wherein said method performs an energy calculation involving the following steps:

a) identification of residues of a monomer that could establish specific interactions with the contiguous monomer;

b) identification of side chains that contact residues that are candidates for mutation;

c) at each residue position is placed each amino acid in a repertoire selected from a set of naturally occurring amino acids in a multiple sequence alignment of members of the same protein family, and any side-chain conformations and amino acids that are not compatible with the rest of the structure are eliminated;

d) all possible pair-wise interactions are explored to eliminate those combinations that are not favourable.

50. A method according to claim 49, wherein said energy calculation is carried computationally, taking into account the properties of the structure, including its atomic contact map, the accessibility of its atoms and residues, the backbone dihedral angles, in addition to the H-bond network and electrostatic network of the protein, the contribution of water molecules making two or more H-bonds with the protein, polar and hydrophobic solvation energies, van der Waals' interactions, van der Waals' clashes, H-bond energies, electrostatics, and backbone and side chain entropies.

51. A method according to claim 50, wherein the method outputs a sequence and/or PDB coordinates corresponding to the best calculated solution.

52. A method according to claim 51, wherein the sequence and/or PDB co-ordinates including the mutations are energy-minimized and the final predicted energies are compared to the reference, wild-type structure in terms of $\Delta\Delta G$ (kcal mol-1).

53. A method for the alteration of the selectivity of a β sheet multimeric cytokine for a target receptor, the method comprising

a) identifying amino acids in the cytokine that are located in the receptor-binding interface as candidates for mutation;

b) discarding residues interacting with amino acids that are conserved among receptors bound by the cytokine protein;

c) discarding residues interacting with the receptor backbone; and

d) substituting each of one or more residues in the cytokine protein for replacement residues that include amino acid side-chain conformations that are predicted to fit into the binding interface with the target receptor so as to provide an increase in binding affinity and selectivity/specificity of the cytokine protein for that target receptor.

54. A β sheet multimeric cytokine whose sequence has been altered by a method according to claim 53 so as to alter its affinity for a particular target receptor.

55. A cytokine according to claim 54, which is mutated at one or more of the positions 131, 269, 130, 160, 218, 220, 149, 155, 214, 195, 191 and 267.

56. A cytokine according to claim 52 or 53, which is a member of the TNF ligand family.

57. A cytokine according to claim 54, which is TRAIL.

58. A cytokine according to claim 57, which has superior selectivity for the DR5 (TRAIL-R2) or DR4 (TRAIL-R1) over the decoy receptors DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4).

59. A cytokine according to claim 57, which has superior selectivity for the death receptor 5 (TRAIL-R2) over selectivity for the death receptor 4 (TRAIL-R1).

60. A cytokine according to claim 58 or 59, which contains one or more of the mutations G131R, D269H, D269K, D269R, R130E, G160K, D218R, G160M, D218Y, D218E, D218K, D218H, I220M, I220H, R149D, R149H, D218F, E155M, T214R, E195R, R191E and D267R.

61. A cytokine according to claim 60, which contains the mutations G160M or D269H.

62. A method for obtaining variants of a β sheet multimeric cytokine with enhanced stability and increased binding affinity and selectivity/specificity for a target receptor comprising the steps of:

a) mutating a residue in a monomer component of the multimeric cytokine protein so as to improve the free energy of the monomer or of the multimeric complex relative to the wild-type unmutated monomer component, wherein said mutated residue is non-conserved between homologous members of the cytokine family, and

b) identifying amino acids in the cytokine that are located in the receptor-binding interface as candidates for mutation, discarding residues interacting with amino acids that are conserved among receptors bound by the cytokine protein, discarding residues interacting with the receptor backbone; and substituting each of one or more residues in the cytokine

protein for replacement residues that include amino acid side-chain conformations that are predicted to fit into the binding interface with the target receptor.

63. A method of treating cancer by exposure of cancer cells to a DR4-specific TRAIL variant in combination with cytotoxic therapies such as ionising radiation and chemotherapy.

64. A method of treating cancer by exposure of cancer cells to a DR5-specific TRAIL variant in combination with cytotoxic therapies such as ionising radiation and chemotherapy.

65. Use of a DR4-specific TRAIL variant in the manufacture of a medicament for the treatment of cancer, wherein the medicament is administered in combination with cytotoxic therapies such as ionising radiation and chemotherapy.

66. Use of a DR5-specific TRAIL variant in the manufacture of a medicament for the treatment of cancer, wherein the medicament is administered in combination with cytotoxic therapies such as ionising radiation and chemotherapy.